

# Suppression of vascular endothelial growth factor expression at the transcriptional and post-transcriptional levels

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## ABSTRACT

Gene expression is regulated at the transcriptional and post-transcriptional levels. Therefore, in order to achieve a high level of silencing, which includes minimizing any residual expression of a target gene, suppression at both the transcriptional and post-transcriptional levels is required. In this study, we describe a new method for highly efficient gene silencing that combines zinc finger protein-mediated transcriptional repression and small interfering RNA (siRNA)-mediated inhibition of post-transcriptional events. To measure the amount of gene expression under various conditions, we used a luciferase reporter gene that was driven by a variety of promoters, including that of the human vascular endothelial growth factor-A (*VEGF-A*) gene. We also measured expression of the endogenous *VEGF-A* gene. Inhibition of gene expression by each of the two individual technologies was effective, but in-depth analyses revealed residual expression of the target gene. The combination of specific zinc finger transcription factors and siRNAs greatly enhanced the silencing of the human *VEGF-A* gene, not only when cells were grown in the presence of normal amounts of oxygen but also under conditions of hypoxic stimulation. These results suggest that a bi-level approach to the silencing of *VEGF-A* expression may be clinically beneficial as part of a cancer treatment protocol.

## INTRODUCTION

A fundamental goal of scientists working in this post-genomic age is the ability to tightly regulate the expression of specific

genes. Designer zinc finger transcriptional regulatory proteins and RNA interference (RNAi) are two key technologies that offer powerful means of achieving this goal.

Gene expression is regulated by two related yet separate events: (i) transcription of the gene into an RNA molecule and (ii) post-transcriptional regulation that includes processing of this precursor RNA to form a mature messenger RNA molecule and translation into a protein. Zinc finger transcription factors regulate expression at the level of transcription, while RNAi functions during post-transcriptional processing. In 1998, Fire *et al.* (1) reported that, in the worm *Caenorhabditis elegans*, double-stranded RNA (dsRNA) molecules inhibit the translation of target mRNAs more efficiently than do individual single-stranded RNAs and the resulting phenotype is apparently inheritable by progeny. The exogenous dsRNA is processed into 19- to 23-nt small interfering RNAs (siRNAs) by the worm enzyme Dicer. Incorporation of these siRNAs into the RNA-induced silencing complex (RISC) is required for the post-transcriptional recognition and subsequent degradation of mRNAs that are homologous to the siRNAs (2). The RNAi phenomenon was observed for a large number of genes either by using dsRNAs that are complementary to the target mRNAs or by screening RNAi libraries in *C.elegans* (1,3,4), *Drosophila* (5), plants (6,7) and mammalian cells (8–10), including the human embryonic kidney cell line HEK293 (10). RNAi-mediated gene silencing at the transcriptional level was reported in limited studies with plants (11–13) and *Drosophila* (14), but this phenomenon has not been clearly observed in mammalian cells. Thus the major function of siRNAs in mammalian cells appears to be to silence gene expression at the post-transcriptional level. RNAi is highly specific, as shown in genome-wide gene expression profiles obtained during inhibition of green fluorescent protein expression (15) and in isoform-specific suppression of mouse vascular endothelial growth factor-A (*VEGF-A*) mRNA (16) by siRNAs.

The C2-H2 class of zinc finger proteins (ZFPs) constitutes one of the most common DNA binding motifs found in

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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eukaryotic transcription factors and provides an ideal scaffold on which to generate zinc finger transcription factors with novel DNA binding specificities (17). We and several other groups have characterized extensively the modularity and specificity of naturally occurring and artificially engineered zinc finger domains (18–22). Advancements in ZFP technology have allowed the production of artificial ZFPs by *in vitro* screening of randomized composite zinc finger domains (19–21) or by mixing and matching pre-characterized zinc finger domains (22). Thus we now have the capability to design specific ZFPs that bind with high affinity to selected DNA sequences and activate or repress target gene transcription, when fused to a transcriptional activation or repression domain, respectively. The ability of ZFPs to selectively regulate transcription of endogenous genes has been reported for mammalian cells (22–26), plants (27), yeast (28) and eubacteria (unpublished data), and accumulating evidence clearly indicates that the ZFP technology is one of the most versatile tools for upregulating or downregulating target gene transcription (23,24). The modular nature of ZFPs has facilitated the generation of libraries that encode diverse ZFP-transcription factors (ZFP-TFs) that interact with specific DNA sequences and regulate gene transcription on a genome-wide scale. In addition, recent studies have shown that ZFP transcription factor libraries can be used to induce particular phenotypes and to identify the genes that are responsible for the phenotype of interest (28,29).

In this paper, we show that ZFP technology and RNAi can be combined to obtain highly efficient silencing of the *VEGF-A* gene. *VEGF-A* is an angiogenic factor that is crucial for both the formation of normal blood vessels and the vascularization and growth of tumors. *VEGF-A* gene expression is stimulated by hypoxic conditions, which manifest in the tumor cells along with tumor growth. Suppression of *VEGF-A* expression by both methods simultaneously almost completely ablated hypoxia-stimulated VEGF induction, suggesting that these methods might be beneficial in the treatment of cancers.

## MATERIALS AND METHODS

### Plasmid constructs for ZFP-TFs, reporter genes and short hairpin RNAs

The cloning of ZFP-TFs pF268, pF121, pF109 and pF435 is described elsewhere (22). The amino acid sequences of four ZFPs are:  $\text{NH}_3^+$ -YVCDVEGCTWKFARSDELNRHKK-RHTGEKPFQCKTCQKFSRSDHLKTHTRTHTGEKPYV-CDVEGCTWKFARSDELNRHKKRH-CO<sub>2</sub><sup>-</sup> [F268],  $\text{NH}_3^+$ -YKCEECGKAQFRQSSHLTTHKIIHTGEKPYKMECGKA-FNRRSHLTRHQRIHTGEKPFQCKTCQKFSRSDHLKTH-TRTH-CO<sub>2</sub><sup>-</sup> [F121],  $\text{NH}_3^+$ -YVCDVEGCTWKFARSDELNR-HKKRHHTGEKPYKCPDCGKSFSQSSSLIRHQRTHTGEKPYKCEECGKAQFRQSSHLTTHKIIHTGEKPYICRKCGRGFS-RKSNLIRHQRT-H-CO<sub>2</sub><sup>-</sup> [F109] and  $\text{NH}_3^+$ -YKCGQCGKFY-SQVSHLTRHQKIHTGEKPFQCKTCQKFSRSDHLKTHTRTHTGEKPYKCGQCGKFYSQVSHLTRHQKIHT-H-CO<sub>2</sub><sup>-</sup> [F435]. To add a transcriptional repression domain [Krüppel-associated box A (KRAB), a segment of the KRAB-A domain from the rat kidney transcription factor kid-1] to an engineered ZFP domain, the DNA encoding the assembled ZFP was subcloned into pLFD-KRAB (Figure 1A) using the EcoRI and

NotI restriction sites, resulting in a construct that encoded the following fusion protein: hemagglutinin tag (HA)–nuclear localization signal (NLS)–ZFP–KRAB. The KRAB repression domain used in these studies contains 63 amino acids ( $\text{NH}_3^+$ -VSVTFEDVAVLFTTRDEWKKLDLSQRSLYREVMLE-NYSNLASMAGFLFTKPKVISLLQQGEDPW-CO<sub>2</sub><sup>-</sup>) that constitute the minimal segment that retains transcriptional repressor activity (obtained from the pTet-tTS vector, Clontech, Palo Alto, CA, USA).

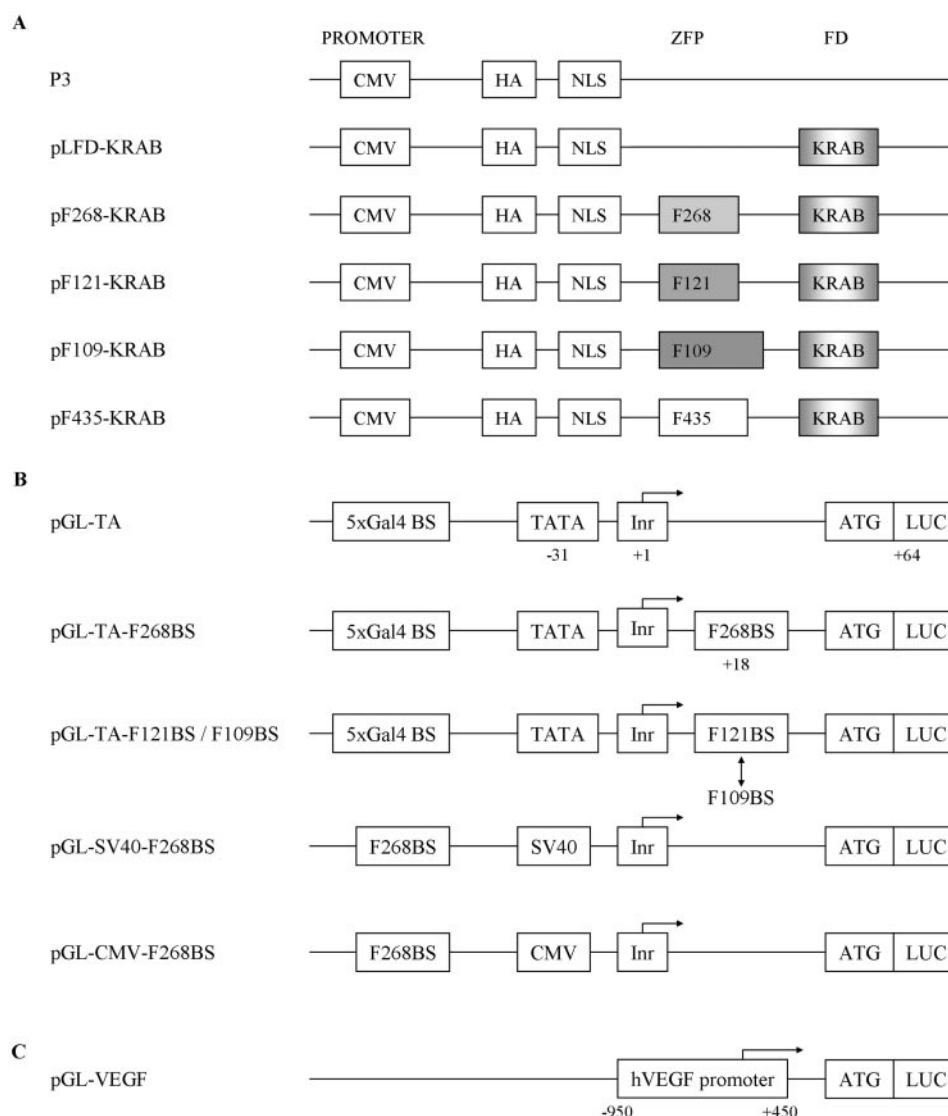
The luciferase reporter construct pGL-TA-ZFPBS and the plasmid encoding the transcriptional activator Gal4-VP16 were described elsewhere (30), except for the following minor modifications to the reporter construct. Briefly, sequences from –124 to –110 from the transcriptional initiation site of pGL3-TATA/Inr+18 (30) were deleted with a KpnI/MluI digest, and the resulting vector was blunt-end ligated. A BglII site was inserted by substituting an A for a G at position +12 from the site of transcription initiation to create pGL-TA (Figure 1B). pGL-TA was cleaved with BglII and HindIII, and zinc finger binding sequences (Table 1) were inserted. To create the dsDNA encoding the zinc finger binding sites, complementary oligonucleotides were synthesized and hybridized by gradual cooling from 90°C to 25°C in 100 mM potassium acetate, 30 mM HEPES KOH pH 7.4 and 2 mM magnesium acetate.

The native human *VEGF-A* promoter DNA (–950 to +450 from the transcription initiation sequence) was amplified by subjecting human genomic DNA to the PCR using specific primers. The amplified human *VEGF-A* promoter DNA was then cloned into the KpnI and XhoI restriction sites of pGL3, yielding pGL-VEGF (Figure 1C). The primer sequences used for *VEGF-A* promoter cloning were: 5'-CGG GGT ACC CCC TCC CAG TCA CTG ACT AAC-3' (forward) and 5'-CCG CTC GAG TCC GGC GGT CAC CCC CAA AAG-3' (reverse).

A vector-based short hairpin RNAs (shRNA) expression system was used to endogenously express shRNA in mammalian cells. An shRNA that binds to and induces the degradation of endogenous *VEGF-A* mRNA in HEK293 cells (thereby repressing expression of the VEGF-A protein) was prepared as follows. Several DNA sequences that encode *VEGF-A* shRNAs were designed, on the basis of the sequence of VEGF-165 mRNA, to target multiple sites of the transcript (GenBank accession no. GI 19909064) and chemically synthesized. The sequences for two representative shRNAs are shown in Table 2. The DNA sequences were cloned into the BamHI/HindIII restriction site of the pSilencer<sup>TM</sup> hygro vector (Ambion, Austin, TX, USA), and the resulting shRNA expression plasmids were then transfected into HEK293F cells (Life Technologies, Carlsbad, CA, USA), which permit a high efficiency of transfection.

### siRNA synthesis and hybridization

Antisense RNA oligonucleotides that were complementary to the translation start site of firefly luciferase mRNA (+153 to +173) (siRNA-luc) were chemically synthesized by Synthetic Genetics (San Diego, CA, USA). The sequences of the sense and antisense RNAs were 5'-CUUACGCUGAGUACUUC-GATT-3' (sense) and 5'-UCGAAGUACUCAGCGUAAG-TT-3' (antisense), as was published by Elbashir *et al.* (10).



**Figure 1.** Constructs used in the experiments described herein. (A) Expression vector constructs for ZFP-KRAB artificial transcriptional repressors. Zinc finger domains were designed and assembled on a p3 vector that included the CMV promoter, HA-epitope and NLS. The resulting zinc finger expression vectors pF268, pF121 and pF435 consist of three zinc finger domains, and pF109 consists of four zinc finger domains. DNA binding domains F268, F121, F109 and F435 were subcloned into pLFD-KRAB, yielding pF268-KRAB, pF121-KRAB, pF109-KRAB and pF435-KRAB. The amino acid sequence of the KRAB transcriptional repression domain is described in Materials and Methods. FD, functional domain. (B) Firefly luciferase reporter constructs with the TATA-minimal promoter (pGL-TA) and five copies of the Gal4-binding sequence (5 × Gal4BS) (pGL-TA-ZFPBS). A single copy of the ZFP binding sequences was introduced at the +18 position relative to the transcriptional initiation site (Inr). The binding sequences for each ZFP are described in Table 1. pGL-SV40-F268BS or pGL-CMV-F268BS are firefly luciferase reporter constructs that contain an SV40 promoter or a CMV promoter, respectively. At the 5' end of the promoter, three copies of a zinc finger binding sequence for F268 were introduced. The binding sequence for F268 is described in Table 1. (C) pGL-VEGF is a luciferase reporter construct that contains the native human VEGF-A promoter sequences (from −950 to +450 relative to transcriptional initiation site). Binding sequences for F121 and F109 are present at positions +434 and −536, respectively.

The double-stranded siRNA molecules were prepared by brief boiling and slow cooling, and were then stored at −80°C until they were used for transfections.

### Cell culture and transfection of 293 cells

HEK293 cells (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (all from Life Technologies, Carlsbad, CA, USA). For the transient transfections, 293 cells ( $2 \times 10^4$  cells/well) were pre-cultured for

24 h in poly D-lysine-coated 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) that contained 100 µl of the medium described above but deprived of antibiotics. For some experiments (e.g. mRNA analysis),  $10^5$  cells/well were cultivated in 24-well culture plates. Unless otherwise indicated, the 293 cells were transfected with a nucleic acid mixture that totaled 0.15 µg (for 96-well culture plates) or 0.5 µg (for 24-well culture plates).

The nucleic acid mixtures included various plasmids: effector plasmids that encode ZFPs (Figure 1A), reporter plasmids encoding firefly luciferase (Figure 1B) and internal

**Table 1.** Zinc finger proteins and their target DNA sequences

ZEP	Amino acid sequence of $\alpha$ -helix <sup>a</sup>	Target DNA sequences
F268	<b>RSDELNR-RSDHLKT-RSDELNR</b>	5'-GCG TGG GCG-3' 3'-CGC ACC CGC-5'
F109	<b>RSDELNR-QSSSLIR-QSSHLTT-RKSNLIR</b>	5'-GAG CGA GCA GCG-3' 3'-CTC GCT CGT CGC-5'
F121	<b>QSSHLTT-RRSHLTR-RSDHLKT</b>	5'-TGG GGG TGA-3' 3'-ACC CCC ACT-5'
F435	<b>QVSHLTR-RSDHLKT-RRSHLTR</b>	5'-GGG CGG GGA-3' 3'-CCC GCC CCT-5'

Bold letters indicate putative DNA-contacting residues.

<sup>a</sup>The amino acid sequences (–1 to 6th residues) in  $\alpha$ -helices of each zinc finger domain using the 1-letter code.

**Table 2.** Oligonucleotide sequences used for the cloning of shRNA expression plasmids and the sequences of the resulting shRNAs

shRNA	Oligonucleotide sequence
shVEGF+64	5'- <b>GATC</b> CCGTGGTCCAGGCTGCACCCC <i>TTCAAGAGA</i> GGGGTGCAGCCTGGGACCACTTTTTTGGAAA-3' <sup>a</sup> 5'- <b>AGCT</b> TTTCCAAAAAGTGGTCCAGGCTGCACCCC <i>TCTCTTGAA</i> GGGGTGCAGCCTGGGACCACTGGG-3' <sup>a</sup> 5'-GUGGUCCAGGCUGCACCCC <i>UUCAAGAGA</i> GGGGUGCAGCCUGGGACCAUUUUUU-3' <sup>b</sup>
shVEGF+338	5'- <b>GATC</b> CCGGCCAGCACATAGGAGAGA <i>TTCAAGAGA</i> TCTCTCTATGTGCTGGCC TTTTGGAAA-3' <sup>a</sup> 5'- <b>AGCT</b> TTTCCAAAAAGGCCAGCACATAGGAGAGA <i>TCTCTTGAA</i> TCTCTCTATGTGCTGGCCGG-3' <sup>a</sup> 5'-GGCCAGCACAUAGGAGAGA <i>UUCAAGAGA</i> <u>UCUCUCCUAUGUGCUGGCCUUUUUU</u> -3' <sup>b</sup>

Bold letters indicate BamHI and HindIII restriction site overhangs.

Italic letters indicate the loop structure.

Underlined are RNA sequences complementary to the target sites.

<sup>a</sup>The oligonucleotide sequences for cloning of shRNAs.

<sup>b</sup>The expected shRNA sequence.

control *Renilla* luciferase (pRL-SV40; Promega, Madison, WI, USA) and plasmids that encode the transcriptional activator Gal4-Vp16 (30). In some experiments, the indicated amounts of siRNA or plasmids that encode shRNA were added to the DNA mixtures prior to Lipofectamine complex formation. Transfection was performed with the Lipofectamine Plus transfection kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Cells were further incubated at 37°C for 48 h in a humid atmosphere with 5% CO<sub>2</sub>.

To measure luciferase expression driven by the native VEGF-A promoter, the Lipofectamine Plus transfection kit was used to transfect 293 cells ( $2 \times 10^4$ ) with the pGL-VEGF reporter and either F121-KRAB or pF109-KRAB at a 1:1 molar ratio (final DNA concentration: 100 ng).

### Luciferase assay

After incubation at 37°C for 48 h after transfection, cultured cells were washed twice with phosphate-buffered saline (PBS) and mixed with passive lysis buffer (30  $\mu$ l; Promega, Madison, WI, USA). Firefly and *Renilla* luciferase activities in the cell lysates were measured in dual wavelength in a TD-20/20 luminophotometer (Turner Designs, Sunnyvale, CA, USA) after sequential addition of the LAR II reagent (40  $\mu$ l), stop buffer (40  $\mu$ l) and the Glo reagent to the lysates (8  $\mu$ l). The Dual-luciferase reporter assay kit (E1960) was purchased from Promega.

### Quantification of VEGF-A mRNA

Because they permit a very high efficiency of transfection, 293F cells (Gibco Life Technologies, Carlsbad, CA, USA),

rather than HEK293 cells, were used to analyze the extent of repression of endogenous *VEGF-A* expression. Cells were maintained under the same conditions used for the HEK293 cells except that non-essential amino acids (0.1 mM; Life Technologies, Carlsbad, CA, USA) were added to the culture media.

For the RT-PCR analysis, 10<sup>5</sup> 293F cells/well were pre-cultured, at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>, for 24 h in 24-well plates containing culture medium (0.5 ml; supplemented with 10% FBS but deprived of antibiotics). The cells were then transfected with DNA using a Lipofectamine Plus transfection kit (Life Technologies). Briefly, a total of 0.5  $\mu$ g of plasmid DNA (i.e. a plasmid that encodes a ZFP, with or without plasmids encoding shRNAs) were mixed with 4  $\mu$ l of the Plus reagent in 25  $\mu$ l DMEM, and then another 25  $\mu$ l of DMEM containing 2.5  $\mu$ l of Lipofectamine reagent was added to the mixture. The culture growth medium was removed from the cells and replaced with 200  $\mu$ l of fresh serum-free DMEM prior to transfection. After 15 min of incubation, the 50- $\mu$ l mixtures described above were then added to each well of the culture plate. Six hours after transfection, DMEM containing 20% FBS (250  $\mu$ l) was added to each culture well, and the cells were grown for an additional 48 h. The cells and culture supernatants were harvested for RT-PCR analysis and an enzyme-linked immunosorbent assay (ELISA).

The cell pellets were lysed in Trizol reagent (Life Technologies) and stored at –80°C until total RNA was extracted. RNA was extracted from the Trizol-lysates according to the manufacturer's instructions (Life Technologies). The reverse transcription reactions were performed with 4  $\mu$ g of total RNA using oligo-dT as the first-strand synthesis primer for mRNA



and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase provided in the Superscript first-strand synthesis system (Life Technologies). To analyze mRNA quantities, 1  $\mu$ l of the first-strand cDNAs generated from the RT reactions were amplified using *VEGF*-specific primers [5'-GTG CAT TGG AGC CTT GCC TTG-3' (forward) and 5'-ACT CGA TCT CAT CAG GGT ACT C-3' (reverse)]. The specific *VEGF-A* mRNA amounts were normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantities calculated by specific amplification using GAPDH-specific primers [5'-CCA TGT TCG TCA TGG GTG TGA-3' (forward) and 5'-CAT GGA CTG TGG TCA TGA GT-3' (reverse)]. Amplification of *VEGF-A*-specific and GAPDH-specific cDNAs were monitored and analyzed in real-time with a Quantitect SYBR kit (Qiagen, Valencia, CA, USA) and a Rotorgene 2000 real-time cyclor (Corbett Research, Mortlake, NSW, Australia), and were quantified using serial dilution of the standards included in the reactions.

### Quantification of secreted VEGF-A protein

The HEK293 cell culture supernatants were centrifuged for 5 min to remove cells and cell debris. VEGF-A protein that accumulated in the culture medium (100  $\mu$ l each) and dilutions of a recombinant human VEGF-A protein standard were analyzed using sandwich ELISA, wherein the supernatant of the culture was incubated with anti-human VEGF-A antibody (AF-293-NA; R&D Systems, Minneapolis, MN, USA), biotinylated anti-human VEGF-A antibody (BAF293; R&D Systems) and streptavidin alkaline phosphatase (SA110; Chemicon, Temecula, CA, USA). The antigen-antibody complex was then incubated with *p*-nitrophenyl phosphate (Sigma, St Louis, MO, USA) dissolved in pNPP buffer (Chemicon; ES011). VEGF-A concentrations in the samples were determined from the absorbance at 405 nm, which was measured with a POWERWAVE(tm) X340 (Bio-TEK Instrument Inc., Richmond, VA, USA).

## RESULTS

### Suppression of reporter gene expression by ZFP-TFs, siRNAs or both

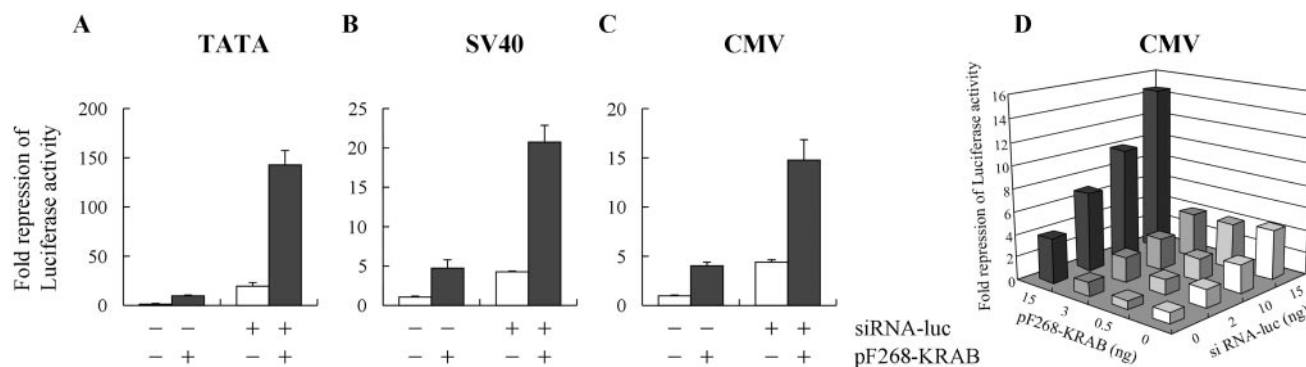
In order to examine whether a ZFP transcriptional repressor and siRNA, each of which targets the same gene, could function additively or synergistically to suppress its expression, we used the firefly luciferase reporter gene under the control of various promoters. First, we tested a well-characterized synthetic promoter (termed TATA) that contains the adenovirus major late promoter TATA box and the murine terminal deoxynucleotidyltransferase initiator (30). So that the effects of a ZFP-TF or siRNA on activated transcription could be studied, a set of five GAL4 binding sites was included upstream of the TATA promoter to generate pGL-TA. For some experiments, as shown in Figure 1B and Table 1, a 9-bp ZFP binding site (5'-GCG TGG GCG-3') was incorporated downstream of the murine terminal deoxynucleotidyltransferase initiator element (pGL-TA-F268BS). The ZFP used in this experiment (termed F268) is a derivative of the well-defined mouse ZFP zif268, in which the third finger of zif268 was replaced with a human zinc finger domain

referred to as RDER (22). F268 is functionally equivalent to the zif268 zinc finger peptide in terms of its DNA-binding affinity and specificity (data not shown). This ZFP was fused to the KRAB transcriptional repression domain, and the plasmid that expressed the resulting ZFP-TF was designated pF268-KRAB (Figure 1A).

HEK293 cells were co-transfected with pF268-KRAB and an expression plasmid encoding the GAL4-VP16 (30) protein (a transcriptional activator), together with the luciferase reporter plasmid containing the appropriate ZFP binding site (pGL-TA-F268BS). The ZFP-TF (F268-KRAB) showed a  $\sim$ 10-fold ( $\pm$ 0.7) repression (i.e.  $\sim$ 90%) of GAL4-VP16-activated transcription from the synthetic promoter. We also transfected 293 cells with pGL-TA-F268BS and with chemically synthesized antisense RNA oligonucleotides that are complementary to the translation start site of firefly luciferase mRNA (siRNA-luc). The siRNA-luc oligonucleotides showed an  $\sim$ 20-fold ( $\pm$ 2.1) repression (i.e. 95%) of GAL4-VP16-activated transcription of the luciferase gene.

Although each reagent (the ZFP-TF and siRNA) on its own was effective at repressing transcription, neither the ZFP-TF nor siRNA-luc was able to achieve complete gene silencing under our experimental conditions. In order to achieve complete suppression of gene expression, we transfected 293 cells with both siRNA-luc and pF268-KRAB, along with the reporter plasmid (pGL-TA-F268BS) and the GAL4-VP16 expression plasmid. Co-transfection of the ZFP-TF-encoding plasmid and siRNA-luc resulted in a dramatic increase in the extent of repression over what was observed with each of the reagents individually. For example, in the presence of siRNA-luc and in the absence of the ZFP-TF expression plasmid, an  $\sim$ 20-fold repression of reporter gene expression was observed relative to the control conditions, while in the presence of both reagents, an  $\sim$ 140-fold ( $\pm$ 15) (99.3%) repression of reporter gene expression was detected relative to the control (Figure 2A).

We also tested the SV40 early promoter and the cytomegalovirus (CMV) immediate early promoter to examine whether ZFP-TFs and siRNAs can repress gene expression synergistically at naturally occurring promoters as well as at the synthetic promoter. As shown in Figure 1B, the 9-bp ZFP (F268) binding sequence was incorporated upstream of the SV40 and CMV promoters. Human 293 cells were co-transfected with the ZFP-TF-encoding plasmid (pF268-KRAB), siRNA-luc or both, together with the SV40 promoter-driven and CMV promoter-driven luciferase reporter constructs. The GAL4-VP16 expression plasmid was not included in these experiments. The ZFP-TF alone showed a moderate 5.0-fold (80%) repression, and siRNA alone showed 4.8-fold (79%) repression of expression from the SV40 promoter relative to the control. Co-transfection of siRNA and the ZFP-TF-encoding plasmid yielded 21-fold (95%) repression of expression from this promoter (Figure 2B) relative to the control. Comparable results were observed with the CMV promoter construct; the ZFP-TF or siRNA alone showed 4.0-fold (75%) or 4.2-fold (76%) repression relative to the control, respectively, whereas the combination of the ZFP-TF and siRNA resulted in a much stronger 15-fold (93%) repression of expression from the CMV promoter relative to the control (Figure 2C). Figure 2D shows a representation of the dose-dependent synergistic effect on luciferase expression of



**Figure 2.** Regulation of luciferase with a combination of siRNAs and F268-KRAB. HEK293 cells were transfected with 50 ng (+) of siRNA, 15 ng (+) of pF268-KRAB, neither effector (–) or both effectors together with (A) the pGL-TA-F268BS reporter (15 ng) plus transcriptional activator Gal4-VP16 (68.5 ng), (B) the pGL-SV40-F268BS reporter (15 ng) or (C) the pGL-CMV-F268BS reporter (15 ng). The *Renilla* luciferase expression plasmid (1.5 ng pRL-SV40) was added in equal amounts to the nucleic acid mixtures. The total amounts of DNA and RNA were brought to 150 ng by the addition of empty vector. Luciferase activities were measured 48 h post-transfection. Fold repression values were obtained after normalization of firefly luciferase activity to *Renilla* luciferase activity then compared with values obtained from control cultures transfected with empty vectors and reporters [pF268-KRAB (–), siRNA (–)]. Results are the mean values and standard errors of three independent experiments. (D) Simultaneous representation of the dose effects of the ZFP-TF and siRNA in the repression of luciferase activity measured in 293 cell cultures co-transfected with varying amounts of plasmids that encode a ZFP-TF (0–15 ng) or siRNA (0–50 ng) together with the reporter pGL-CMV-F268BS. Fold repression values were obtained after normalization of firefly luciferase activity to *Renilla* luciferase activity then compared with values obtained from control cultures [0 ng pF268-KRAB + 0 ng siRNA]. The total amount of nucleic acid was calibrated to a final amount of 100 ng with the empty vector pLFD-KRAB. Results are the mean values of three independent experiments.

pF268-KRAB and siRNA with the strongest promoter-driven reporter construct (CMV).

To confirm that the observed repression of luciferase reporter gene expression by the ZFP-TF was dependent on the ZFP-TF target DNA binding sequence, we co-transfected 293 cells with the ZFP-TF-encoding plasmid (pF268-KRAB) in combination with one of three reporter plasmids: pGL-TA-F268BS, pGL-TA-F121BS or pGL-TA-F109BS, each of which contains different ZFP binding sequences as shown in Table 1. Repression of reporter gene expression by F268-KRAB was observed only when the reporter contained the matching target ZFP binding site (data not shown). We also have confirmed that siRNA-luc specifically silenced reporter gene expression with our promoter systems; siRNA-luc did not repress luciferase expression when the siRNA target sequence was mutated (data not shown). These data indicate that the suppressive activities of the ZFP-TF and siRNA used in this series of the experiments are specific for the well-characterized targets of the two reagents.

### The ZFP-TF/siRNA combination strengthens silencing of a *VEGF-A* promoter-driven reporter gene

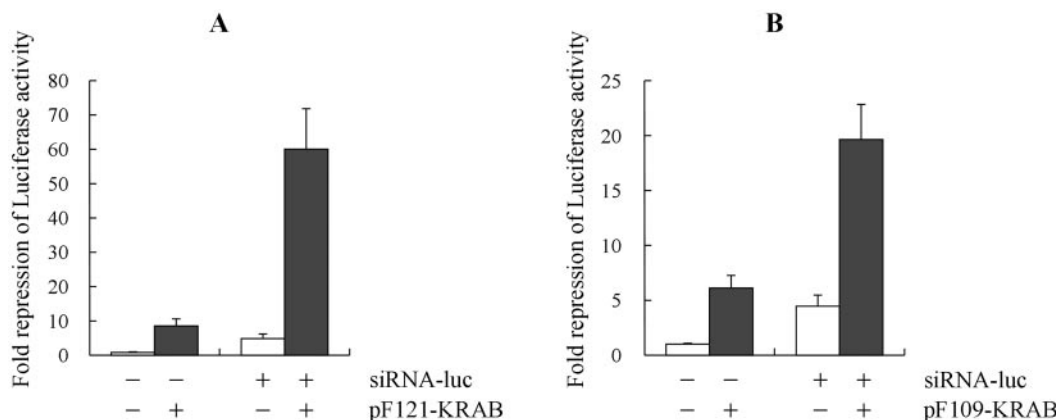
We sought to assess the synergistic gene silencing effect of ZFP-TFs and siRNAs using a luciferase reporter plasmid that contained the native human *VEGF-A* promoter region (–950 to +450 relative to transcriptional initiation site). We used two ZFP constructs (termed F121 and F109, which are composed of 3 and 4 human zinc finger domains, respectively) designed to recognize specific sequences in the *VEGF-A* promoter. We have previously shown that these ZFPs, when fused to a transcriptional activation domain, stimulate transcription of the endogenous *VEGF-A* gene as well as a reporter gene under the control of the *VEGF-A* promoter (22). Here, we sought to generate and test artificial transcription factors that suppress *VEGF-A* gene expression by fusing these ZFPs to the KRAB

domain (Figure 1A). HEK293 cells were co-transfected with a luciferase reporter plasmid containing the *VEGF-A* promoter (pGL-VEGF) and either the F121-KRAB or the F109-KRAB expression plasmid. The ZFP-TFs, F121-KRAB and F109-KRAB, individually suppressed reporter luciferase activity 8.7-fold ( $\pm 2.0$ ) and 6.1-fold ( $\pm 1.2$ ), respectively (Figure 3A and B; see the (–) siRNA groups) compared with the control [(–) siRNA and (–) ZFP]. siRNA (siRNA-luc) alone also yielded a moderate level of gene silencing, i.e. 4.5-fold ( $\pm 1.0$ ) suppression of the same reporter gene (pGL-VEGF) relative to the control [(–) siRNA and (–) ZFP]. As expected, dramatic suppression of luciferase expression was observed when the ZFP-KRAB plasmids were co-transfected with siRNA. Specifically, F121-KRAB and F109-KRAB yielded an  $\sim 61$ -fold ( $\pm 12$ ) (i.e.  $\sim 98.3\%$ ) and an  $\sim 20$ -fold ( $\pm 3.2$ ) ( $\sim 94.9\%$ ) repression of *VEGF* promoter-controlled expression of the luciferase reporter gene, relative to the control, when combined with siRNA.

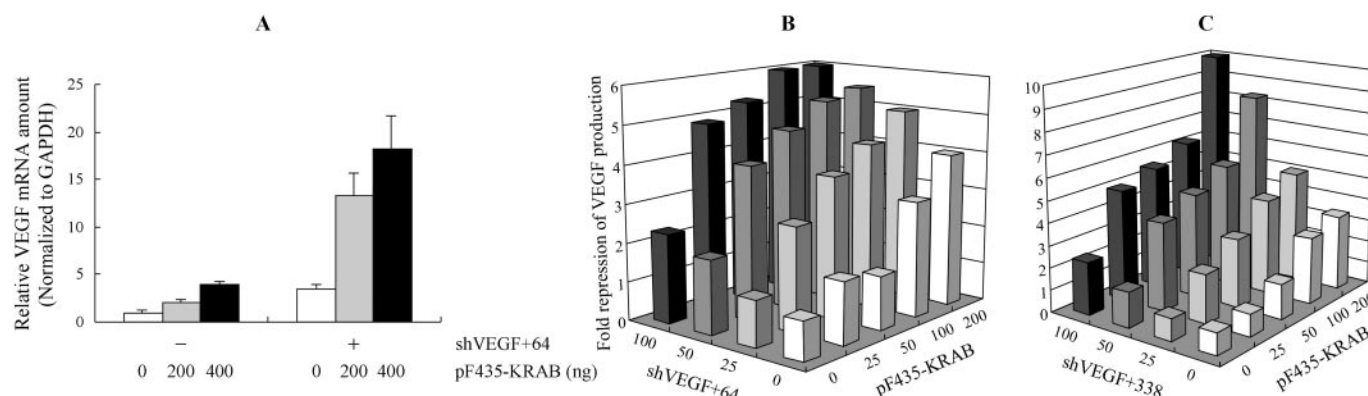
It is interesting to note that, for all four promoters we tested, the fold-repression of the reporter gene with both the ZFP-TF and siRNA is approximately equal to the product of the fold-repression with the ZFP-TF alone and that with siRNA alone. For example, siRNA or the ZFP-TF gave 4.2-fold or 4.0-fold repression of expression from the CMV promoter (pGL-CMV-F268BS), respectively, whereas the two reagents in combination gave  $\sim 15$ -fold repression (Figure 2C). This observation implies that transcriptional repression by ZFP-TFs and gene silencing with siRNA occur by independent mechanisms. We expect that ZFP-TFs in combination with siRNAs should give much stronger repression of most, if not all, target genes than do ZFP-TFs or siRNAs alone.

### RNAi and ZFP-TFs synergistically suppress endogenous *VEGF-A* gene expression

To assess whether RNAi and ZFP-TFs are capable of silencing endogenous human *VEGF-A* gene expression, we tested



**Figure 3.** Synergistic inhibitory effect of ZFP-TFs and siRNAs on luciferase production driven by the native human VEGF promoter. Two hundred and ninety-three cells were transfected with a luciferase reporter construct that contained the native human VEGF-A promoter together with either (A) 30 ng of pF121-KRAB (+), 50 ng of siRNA (+) or both, or (B) 30 ng pF109-KRAB (+), 50 ng siRNA (+) or both. Fold repression values were obtained by comparing repressed amounts of luciferase activity with that measured in cultures that had been transfected with empty vector (–/–) and reporter (pGL-VEGF) only [0 ng pF121-KRAB or pF109-KRAB, 0 ng siRNA]. Results are the mean values and standard errors of three independent experiments.



**Figure 4.** Synergistic repression of endogenous VEGF-A at the mRNA and protein level. 293F cells ( $10^5$  cells/24-well plate) were co-transfected with increasing amounts of a plasmid that encodes a ZFP-TF [pF435-KRAB; 0, 200, 400 ng] and an shRNA-encoding plasmid [shRNA+64; 0(–), 100 ng(+)]. Total transfected DNA was calibrated to a final amount of 500 ng with the addition of the pLFD-KRAB empty vector. VEGF-A mRNA was analyzed by RT-PCR using specific primers (see Materials and Methods). The amounts of VEGF mRNA were normalized to the amounts of GAPDH mRNA from the same reverse transcription product, then fold repression values were calculated by dividing normalized values from control cultures by that of effector-treated cultures (A). Results are the mean values and standard errors of three independent experiments. Also shown are simultaneous representations of the fold repression of secreted VEGF-A protein obtained from 293F cell cultures that had been co-transfected with various combinations of ZFP-encoding plasmids and either shRNA+64 (B) or shRNA+338 (C) and incubated for 72 h. Results are the mean values of three independent experiments.

several ZFP-TFs that were designed to bind to sites in the *VEGF* promoter. Unfortunately, F121-KRAB and F109-KRAB, which showed strong repression in the luciferase reporter assay, were not able to significantly suppress the expression of the endogenous *VEGF* gene (data not shown). These ZFP-TFs may not bind to the target sites in the chromatin state in cells. Thus we used another ZFP, i.e. F435, for the regulation of the endogenous gene. F435 is the DNA binding moiety of a ZFP-TF that was shown to activate *VEGF-A* expression in human cells (22). In the current study, F435 was fused with the KRAB transcriptional repression domain. Snowden *et al.* (31) reported previously that a ZFP-TF composed of engineered zinc finger domains and the KRAB domain was successful in suppressing *VEGF-A* expression in 293 cells and a highly tumorigenic glioblastoma cell line, indicating that ZFP-mediated *VEGF-A* suppression has therapeutic potential for cancer treatment.

For convenience of design and manipulation, we used an expression vector system that yields shRNA that induces *VEGF-A*-specific RNAi in mammalian cells. A series of U6 promoter-driven shRNAs were designed to target human *VEGF-A* mRNA, the most potent being shVEGF+64 and shVEGF+338, which encode RNAs that can form partial double-stranded hairpin structures. shVEGF+64 and shVEGF+338 contain 21-nucleotide sequences that are complementary to *VEGF-A* mRNA beginning at positions +64 and +338 within coding region, respectively (Table 2).

We found that endogenous *VEGF-A* mRNA quantities measured by real-time RT-PCR were reduced up to 4.0-fold (75% reduction) by F435-KRAB (400 ng plasmid) and 3.5-fold (71% reduction) by shVEGF+64 (100 ng plasmid), compared with controls (Figure 4A). Although both reagents repressed *VEGF-A* expression significantly, neither did so completely, even if the amounts of transfected plasmids



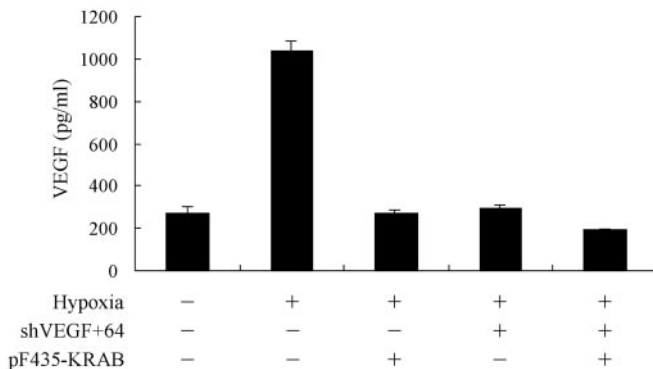
were further increased (data not shown). Co-transfection of plasmids encoding F435-KRAB and shVEGF+64 into 293 cells gave an 18-fold (94%) reduction in the level of *VEGF-A* mRNA compared with controls (Figure 4A). The residual *VEGF-A* mRNA might be expressed from non-transfected cells, which constituted ~5–10% of the population, as calculated from lacZ staining of parallel cells after transfection (data not shown). This suggests that co-transfection of the ZFP-TF and siRNA leads to almost complete silencing of the *VEGF-A* gene in 293 cells.

Next, we performed an ELISA using human VEGF-specific antibodies to quantify the amount of VEGF-A protein present in the culture media. When cells were transfected with the F435-KRAB expression plasmid, the VEGF-A concentration in the media was decreased in a dose-dependent manner (Figure 4B and C; see the shRNA: 0 ng groups). Likewise, the plasmids expressing shRNAs (shVEGF+64 and shVEGF+338) individually showed dose-dependent suppression of VEGF-A protein production (Figure 4B and C; see the pF435-KRAB: 0 ng groups). The strongest repression of VEGF-A protein production with shRNA alone was merely 2.4-fold relative to controls under our experimental conditions. These results were in agreement with the amount of *VEGF-A* mRNA present, as analyzed by RT-PCR experiments.

We then assessed the effect of both the ZFP-TF and shRNA on VEGF-A protein production. Cells were transfected with the two plasmids encoding F435-KRAB and shRNA, and the VEGF-A level was measured by ELISA. A dose-dependent reduction in VEGF-A protein concentration was detected in the presence of increasing amounts of both the F435-KRAB and shRNA expression plasmids; inhibition of VEGF-A secretion reached a maximum of 83% (shRNA+64 plasmid, 100 ng and ZFP-TF plasmid, 200 ng) or 91% (shRNA+338 plasmid, 100 ng and ZFP-TF plasmid, 200 ng), compared with culture supernatants obtained from cells transfected with the control parental vector (Figure 4B and C). The level of inhibition reached by the combined reagents constitutes nearly complete silencing when we take into account the transfection efficiency [~90–95% under our experimental conditions (data not shown)].

#### The combination of ZFP-TFs and shRNAs inhibits hypoxia-induced *VEGF-A* activation

We next performed a set of experiments to determine whether the VEGF-A production-suppressing shRNAs and ZFP-TFs could down-regulate VEGF-A expression in the presence of the hypoxia-inducing agent cobalt chloride ( $\text{CoCl}_2$ ), a strong stimulator of VEGF-A expression in cultured cells. Cells were transfected with plasmids encoding F435-KRAB and/or shVEGF+64, and incubated for 48 h. To induce hypoxic conditions, some cultures were treated with  $\text{CoCl}_2$  during the last 7 h of incubation, after which the VEGF-A concentration was measured by ELISA. Under non-hypoxic conditions, cells secreted 270 ( $\pm 25$ ) pg/ml of VEGF-A in the absence of the ZFP-TF and shRNA, and the VEGF-A amount was increased to 1040 ( $\pm 48$ ) pg/ml under hypoxic conditions. Hypoxia-induced VEGF-A production was reduced independently by F435-KRAB or shVEGF+64, resulting in the secretion of 270 ( $\pm 12$ ) pg/ml or 300 ( $\pm 12$ ) pg/ml of VEGF-A, respectively; these amounts of VEGF-A were similar to those detected in



**Figure 5.** Inhibition of hypoxia-induced VEGF activation by ZFPs and shRNAs. 293F cells were transfected with a plasmid encoding a ZFP-TF (pF435-KRAB), an shRNA expression vector (shVEGF+64) or both, and then cultivated for 48 h. During the last 7 h some cells were exposed to  $\text{CoCl}_2$  (800  $\mu\text{M}$ ) to induce hypoxia (+). Secreted VEGF-A protein was analyzed by ELISA. Non-hypoxic (–) or hypoxic (+) cell cultures transfected with empty vectors (–shRNA+64, –F435-KRAB) served as controls. Results are the mean values and standard errors of two independent experiments.

the non-hypoxic culture (Figure 5). The amount of VEGF-A produced in the cultures co-transfected with plasmids encoding the ZFP-TF and shVEGF+64 was further reduced to 190 ( $\pm 5.1$ ) pg/ml. The residual amount of VEGF-A might come mostly from hypoxia-stimulated, non-transfected cells, which constituted ~10% of the cell population present in the culture.

Taken together, the combined effect of the ZFP-TF and shVEGF+64 on *VEGF-A* expression under both normal and hypoxic conditions suggests that silencing of the *VEGF-A* gene using these reagents might be effective for inhibiting hypoxia-stimulated tumor angiogenesis under pathological conditions *in vivo*.

## DISCUSSION

Regulation of gene expression is orchestrated at both the transcriptional and post-transcriptional levels. Two equally effective technologies, inhibition of gene expression with artificial ZFP-TFs and RNAi, provide the means by which to intervene with each of these processes: ZFP transcriptional repressors down-regulate gene expression at the transcriptional level, and siRNA does so at the post-transcriptional level.

Growing evidence has emphasized the specificity and versatility of ZFPs in modulating the expression of myriad target genes. Recently, Tan *et al.* showed that regulation of a single target gene within the entire human genome is achievable using a 6-finger ZFP transcriptional repressor that recognize an 18-bp site in the genome (32). The ability of ZFPs to activate or repress target gene expression was also exploited in a recent report, where the method was used to identify novel functional relationships among genes (33).

However, the ZFP-TF and siRNA technologies have some serious limitations. It is hard to achieve >10-fold repression of target gene expression with siRNA alone (34), although in some isolated siRNA has been shown to yield almost complete suppression of expression of series of *Drosophila* genes (35) or suppression of replication of variant HIV-1 from Magi cells



when they were co-transfected with siRNA (36). ZFP repressors usually yield a 2- to 10-fold repression of target mRNA expression relative to controls (31,32,37). For many practical applications and for certain research objectives, these moderate levels of gene suppression are sufficient. For other applications, however, stronger repression is desired.

Unlike RNAi, the construction of ZFP-TFs is still a specialized and sophisticated process. However, we note that several groups reported successful applications of this approach in the regulation of diverse genes (22–27,31–32,37–42).

In the present study, we clearly show that combining ZFP-mediated transcriptional regulation and RNAi-mediated post-transcriptional regulation of gene expression leads to a dramatic increase in the extent of gene suppression. Our results with gene expression systems under the control of various promoters and with the endogenous human *VEGF-A* gene strongly suggest that this method is applicable to almost any gene. For example, efficient suppression of *VEGF-A* expression may be useful for the treatment of diseases such as cancer and age-related macular degeneration (AMD) (43).

In addition, these two independent tools for gene regulation, when used in combination, should be of great help in functional genomic studies. RNAi tends to induce up-regulation of interferon-stimulated genes (44) and thus to elicit non-specific effects unrelated to the targeted gene of interest (45). Although single-gene specificity within the human genome has been demonstrated with ZFP-TFs that recognize an 18-bp DNA site (32), ZFPs, especially those that recognize 9- to 12-bp DNA sites, appear to alter the transcript levels of dozens of other genes that are not predetermined targets (22). Considering these so-called ‘off-target’ effects that are associated with RNAi and ZFP-TFs, use of these two independent technologies should yield more robust results than either method alone. Thus researchers should be able to assign gene functions with greater confidence. One may also expect that synergistic suppression of a gene of interest via the combined effects of siRNAs and ZFP-TFs would lead to a more severe change in a phenotype of interest. This characteristic would then allow dose–response modes of investigation.

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